Autoradiography and Immunofluorescence Combined for Autecological Study of Single Cell Activity with Nitrobacter as a Model System

by

C. B. Fliermans
E. I. du Pont de Nemours and Company
Savannah River Laboratory, Environmental Transport Division
Aiken, South Carolina 29801

and

E. L. Schmidt
Departments of Microbiology and Soil Science
University of Minnesota
Minneapolis, Minnesota 55455

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ABSTRACT

Specific detection of a particular bacterium by immunofluorescence was combined with estimation of its metabolic activity by autoradiography. The nitrifying bacteria Nitrobacter agilis and N. winogradskyi were used as a model system. Nitrobacter were incubated with $NaH^{14}CO_3$ and $^{14}CO_2$ prior to study. The same preparations made for autoradiograms were stained with fluorescent antibodies specific for the Nitrobacter species. Examination by epifluorescence and transmitted dark-field microscopy revealed Nitrobacter cells with and without associated silver grains. Direct detection and simultaneous evaluation of metabolic activity of Nitrobacter was demonstrated in pure cultures, in a simple mixed culture, and in a natural soil.

INTRODUCTION

The fluorescent antibody (FA, immunofluorescence) technique provides a highly selective, sensitive, and direct method for autecological study of specific microorganisms in natural environments. Along with the unique advantages of the technique in permitting the simultaneous detection, identification, and enumeration of a particular bacterium in wide variety of ecosystems, is the disadvantage that dead or inactive cells are stained with FA along with active living cells (12). Dead cells are probably scavenged from natural environments rather rapidly, as with Rhizobium japonicum (2) but certain senescent or starved cells may well persist at low activity for extended periods (3). For many autecological studies it may be necessary to assess relative metabolic activities of the cells observed.

Autoradiography has been used in both pure and mixed cultures to estimate cellular metabolic activity (4, 9, 10, 17). Cells are exposed to an appropriate substrate which carries a radioactive label, and only cells that are active metabolically can incorporate the radioactivity. Radioactivity is detected by covering the specimen with a sensitive silver bromide photographic emulsion, which is exposed slowly by beta radiation emitted from active cells. The radiation produces a latent image which on development forms silver crystals that are readily visible. The unexposed crystals are dissolved from the emulsion during fixation leaving a pattern of silver grains which can be seen with appropriate microscopy as a record of cellular or subcellular activity. A major inherent limitation of the technique is the inability to identify the radioactive organisms beneath the nuclear emulsion in all but the special cases of a pure culture, or of morphologically distinctive cells.

We describe a procedure which combines immunofluorescence and autoradiography to allow the simultaneous observation and recognition of a particular

bacterium while assessing its activity with respect to a given isotope in both cultural and natural systems.

MATERIALS AND METHODS

Growth of Nitrobacter agilis (ATCC 14123) and N. winogradskyi and preparation of their specific fluorescent antibodies were as described (6).

In pure culture experiments cultures were grown in shaken flasks at room temperature in the presence of ^{14}C-$NaHCO_3$ (50 mCi/mM, specific activity) at 1µc/ml final concentration. Growth curves were determined by direct microscopic count of FA-stained cells on membrane filters (1). For combined immunofluorescence-autoradiography, 0.01 ml samples were taken throughout the growth cycle and placed on frosted end glass microscope slides previously washed with alcohol and flamed. Each slide was air dried and gently heat fixed. Unincorporated ^{14}C-$NaHCO_3$ was washed free by transferring the slides consecutively through three buffered saline (pH 7.2) washes of five minutes each. Slides were again air dried and autoradiograms were prepared.

Four replicate glass microscope slides with the heat fixed and washed bacteria were dipped in Kodak NTB-2 nuclear track emulsion diluted to a final concentration of 1:3 with filtered sterilized distilled water. All manipulations involving NTB-2 emulsion were performed in total darkness, without the use of a safe light. Emulsions were stored in the dark at 4°C. The emulsion was heated to 43°C in a constant temperature water bath 30 minutes prior to dipping and freed of air bubbles (11). Slides were immersed in the emulsion in a reproducible manner and immediately placed horizontally on a cold metal tray in order to gel the molten emulsion prior to drying. The metal tray was held in a light tight chamber kept at 45% relative humidity (16), and the slides were allowed to dry for 45 minutes. Dry slides were placed in black plastic slide boxes containing an indicator desiccant, boxes were sealed with black electricians tape, and exposed to the radiation at room temperature for 2 to 10 days. After appropriate exposure times, the slides were developed for 30 seconds at 23°C in Kodak D-19 developer diluted

to a final concentration of 1:3 with tap water, fixed in 30% sodium thiosulfate for two minutes, washed in running tap water for 15 minutes, and air dried. The slides were then stained through the developed emulsion with the appropriate FA as previously described (14).

The $^{14}CO_2$ incorporation experiments were carried out in a soils system enriched for Nitrobacter. Mammoth Cave sediments from Mammoth Cave National Park in Kentucky known to have populations of Nitrobacter sp. were leached free of nitrates and treated with 0.1 mg NO_2^--N/ml. After appropriate incubation the populations of Nitrobacter increased from 4.8×10^3 to 3.6×10^6/gm of sediment as measured directly by quantitative FA techniques (1, 13). The enriched sediment was placed in a glass tumbler and plastic disposable syringe barrels (3cc) were truncated, weighed and inserted into the sediments. The top of the syringe was sealed with a septum and $^{14}CO_2$ (2.4×10^6 cpm) was injected. Preparation of $^{14}CO_2$ and measurements of the levels injected were described by Smith et al. (15). Soil cores were removed after 24, 72, and 168 hours, weighed, and transferred to flasks. The soil suspension was dispersed, soil particles were flocculated from the suspension, and the supernatant containing the Nitrobacter was concentrated onto 0.45 μm membrane filters (1, 13). The organisms were scraped from the filter, resuspended in 0.5 ml phosphate buffered saline, and 0.1 ml of that suspension was placed on glass slides, air dried, heat fixed and used for autoradiogram preparations.

Fluorescent antibodies attached to Nitrobacter cells and autoradiographic silver grains were viewed simultaneously using a Zeiss Universal microscope with a combination of epifluorescence and transmitted dark field illumination. The reflected system for fluorescence used an OSRAM HBO 200/w 2 super pressure mercury lamp, Fl 500 reflector, BG 38 and two FITC exciter filters, and a #50 barrier filter. Silver grains were observed with subdued transmitted white light through an oil immersion dark field condenser. Epifluoresence and dark field microscopy were carried out with two oil immersion objectives

with adjustable iris diaphragms: a 40X, 1.0 N.A. Planapochromat, and a 100X, 1.25 N.A., Planapochromat. Photomicrographs were taken with GAF color film (200 ASA) using 20 second exposures. Since the silver grains and fluorescing cells are in different focal planes, some of the micrographs emphasize the silver grains while others focus on the Nitrobacter cells.

RESULTS

Preliminary experiments showed that certain minimum developing times were necessary to obtain an adequate density of silver grains above or in contact with active individual cells but that prolonged developing time led to high background density of silver grains not associated with radioactivity. Based on the relationsnips shown in Figure 1, developing times between 30 and 60 seconds were suitable with acceptably low background levels of 0.6 to 1.5 silver grains per 1000 μm^2. Data reported in Figure 1 were derived from slides exposed in the dark for a 5-day period; essentially the same results were obtained for preparations exposed for two days and for seven days.

Pure Cultures

The proportion of active cells (those with associated silver grains) to inactive cells (no associated silver grains) was determined at various times in the growth cycle of N. agilis using combined FA and autoradiography, together with FA membrane filter counts to follow single cell activity with population changes. Results shown in Figure 2 indicate that log phase cells were not uniformly active. The highest percentage of active cells, 84, occurred at 22 hours which corresponded to early log phase, while samples taken both before and after this time had substantially lower proportions of active cells. A similar experiment (Figure 3) showed that N. winogradskyi reached about the same maximum percentage of active cells, but much later in the growth cycle. The highest proportion of active N. winogradskyi cells was 88% at 72 hours, during mid-log to later log growth. Background levels for the autoradiograms in both of these experiments ranged from 0.91 to 1.14 silver grains/1000 μm^2.

A series of photomicrographs is presented in Figure 4 to help visualize active and inactive cells in the combined FA-autoradiographic preparations.

Unfortunately, the distinctions made easily under the microscope cannot be relfected in the photomicrographs. When viewed by epifluorescence microscopy, the bacteria fluoresce green whereas the silver grains appear yellow-orange when the transmitted darkfield lighting system is used. Since the autoradiographic emulsion overlays the specimens bacterial cells are in different focal planes. Thus, some of the photomicrographs show the bacteria in focus with rather diffuse silver grains above, and others show the silver grains in focus with the cells seen dimly below. Release of radioactive debris into the medium and subsequent fixation on the slides along with cells accounted for some of the background grains (Figure 4D). There was a progressive increase in background levels of silver grains with incubation time while the proportion of active cells decreased.

Some effects of the concentration of the specific energy source on autoradiograms of N. winogradskyi are reflected in Figure 5. The concentration of nitrite nitrogen usually used for pure culture growth, 1.4 g sodium nitrite/liter (Figure 5A) and a much higher concentration of 10.0 g/liter (Figure 5C) both resulted in less intensive silver grain development around cells and less radioactive extracellular material in the background than did the intermediate concentration of 5.0 g/liter (Figure 5B). Although cells grown at the latter concentration showed a great deal of activity, non-radioactive cells as indicated by the arrow (Figure 5B) still are readily distinguishable. These effects of substrate concentration on silver grain development were repeatable for both N. winogradskyi and N. agilis.

Mixtures of Pure Cultures

The use of combined FA-autoradiography in a simple mixed culture system to detect the kind and activity of cells is shown in Figures 6 and 7. Growth and activity of N. agilis in the presence of N. winogradskyi after inoculation with approximately equal numbers of each is given in Figure 6. When compared

to the behavior of N. agilis in pure culture (Figure 2) it appears that about the same maximum percentage of active cells was reached at about the same time of incubation (23 hours) but that the growth rate dropped off rapidly after only about 30 hours. N. winogradskyi in the mixed culture (Figure 7) resembled very closely the pure culture in both the maximum percentage of active cells (90) and the stage of growth with maximum activity (mid-log). The final population density of N. winogradskyi was greater than that of N. agilis by more than 1 log. These data are in accord with a previous study (6) in which N. winogradskyi out-grew N. agilis, and reached a higher population density when both were grown together.

Mixed Culture Natural Environment

The capability to pick out a single species and evaluate its metabolic activity in a complex natural environment is shown in Figure 8. Introduction of gaseous $^{14}CO_2$ for 72 hours into an actively nitrifying soil from Mammoth Cave National Park resulted in uptake and labeling of about 40 per cent of the measured population of 3.6×10^6 Nitrobacter species per gram of cave sediment. Although microorganisms other than Nitrobacter species were obviously present on the FA-autoradiograms, nearly all of the cells with related silver grain activity were Nitrobacter cells as evidenced by specific immunofluorescence. Figure 8 reflects the problem of distinguishing between silver grains and particulate material in the examination of soil or sedimentary systems, since soil particles and silver grains scatter light in a similar manner. Again the resolution is much easier with the microscope than is indicated by the photomicrographs, since the silver grains are of uniform size and can be confirmed by using phase microscopy rather than darkfield microscopy for particulate preparations.

DISCUSSION

The chemoautotrophic nitrifiers N. agilis and N. winogradskyi proved to be excellent model systems for combining immunofluorescence and autoradiography for several reasons. Fluorescent antibodies for the two species were highly reactive and specific (6). As autotrophs these bacteria incorporated ^{14}C-$NaHCO_3$ rather selectively, and techniques used to study incorporation of labeled carbon in other chemoautotrophs (7) were applicable. Their small dimensions and nondescript morphology constitute a rigorous test for combined FA-autoradiography. Finally, the nitrifiers are legitimate objects of autecological study in view of their importance in nature. The data demonstrate the effective application of the combined techniques at the pure culture level, the two-culture level, and at the level of the complex mixed culture natural environment. It may be expected that similar success can be achieved with equally nondescript heterotrophic bacteria in the face of a less selective labelled substrate so long as adequately specific fluorescent antibodies are available.

The experiments with the two cultures studied individually and then together in a simple mixed culture situation were designed mainly for purposes of development of the technique. Beyond the demonstration that active cells could be distinguished from inactive cells for a given species, the data (Figures 2, 3, 6, 7) reflect distinct physiological differences between the N. agilis and N. winogradskyi cultures used. The differences were most apparent in the mixed culture where N. winogradskyi exhibited its fastest growth rate, whereas N. agilis was able to grow only 2 to 3 generations before reaching stationary phase. Surprisingly slight differences were noted in the maximum proportion of labelled cells irrespective of species or experimental conditions of growth. More surprising is the sharp decline following these maxima for each culture

under all circumstances, since the proportion of active cells dropped off while the cultures were still in log phase. Such findings may have resulted from a depletion of labeled bicarbonate around mid-log, but this possibility was not tested at concentrations higher than the 1μc/ml used throughout. Alternatively, many post mid-log cells may have incorporated less than detectable levels of radiation, since Laudelout et al. (8) found that N. winogradskyi used substrate free energy most efficiently during early exponential growth.

Autoradiograms as used in this study with ^{14}C substrate and Kodak NTB-2 emulsion have certain characteristics that may complicate their interpretation with respect ot the juxtaposition of cells and silver grains. The NTB-2 emulsion diluted 1:3 forms a layer of 2-3 micrometers based on calculations from Rogers (11) and Falk and King (5), and would be less dense than the Ilford G-5 emulsion mentioned by Rogers (11) in his calculations of path lengths for beta particles. The majority of the ^{14}C particle energy is about 20 keV , which have mean track lengths of 2.9 μm in the Ilford emulsion (11). Thus, the efficiency of capturing and recording ^{14}C beta particles in NTB-2 emulsion is relatively low. Although a greater emulsion thickness would increase this efficiency, it would also obscure fluorescing bacteria.

Immunofluorescence and autoradiography combined as a single technique shows distinct promise in providingthe microbial ecologist with a sensitive means of evaluating single cell activity. The possibility of using the approach in complex natural environments to follow a particular bacterium of special physiological interest is especially attractive and worthy of further attention.

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List of Legends

Figure 1. Autoradiographic background levels of NTB-2 emulsion exposed for 5 days at room temperature then developed at 23°C.

Figure 2. Semi-logarithmic plot of growth and relative activity measurements of *N. agilis* in pure culture. *Nitrobacter* medium was amended with 1 µc/ml $NaH^{14}CO_3$ and samples for activity and growth were taken during the growth cycle.

Figure 3. Semi-logarithmic plot of growth and relative activity measurements of *N. winogradskyi* in pure culture. *Nitrobacter* medium was amended with 1 µc/ml $NaH^{14}CO_3$ and samples for activity and growth were taken during the growth cycle.

Figure 4. Photomicrographs of *N. agilis* taken during various stages of growth showing the combined techniques of FA and autoradiography.

a. 3 hrs. growth
b. 22 hrs. growth
c. 46 hrs. growth
d. 100 hrs. growth

Notations are: b = background silver grains; ac = active cell; ic = inactive cell; scale bar, 10 µm.

Figure 5. Photomicrographs of autoradiograms of *N. winogradskyi* taken after 68 hours of growth in the presence of 1 µc/ml $NaH^{14}CO_3$ with various levels of $NaNO_2$.

a. 1.4 mg $NaNO_2$/ml, normal concentrations;

b. 5.0 mg $NaNO_2$/ml;

c. 10.0 mg $NaNO_2$/ml

Notations are: b = background silver grains; ac = active cell; ic = inactive cell; scale bar, 10 µm.

Figure 6. Semi-logarithmic plot of growth and relative activity measurements of *N. agilis* in a mixed culture with *N. winogradskyi*. Initial populations were approximately equal. *Nitrobacter* medium was amended with 1 µc/ml $NaH^{14}CO_3$ and samples for activity and growth determinations were taken during the growth cycle.

Figure 7. Semi-logarithmic plot of growth and relative activity measurements of *N. winogradskyi* in a mixed culture with *N. agilis*. Initial populations were approximately equal. *Nitrobacter* medium was amended with 1 μc/ml $NaH^{14}CO_3$ and samples for activity and growth determinations were taken during the growth cycle.

Figure 8. Autoradiography and immunofluorescence combined to examine activity of individual cells in a natural environment. Photomicrograph shows active and inactive cells of *Nitrobacter* sp. in a preparation of a Mammoth Cave sediment. *N. agilis* and *N. winogradskyi* fluorescent antibodies were combined for immunofluorescent staining. Notations are: b = background silver grains; ac = active cell; ic = inactive cell; s = soil particle; scale bar, 10μm